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# Platelet-leucocyte aggregation is augmented in cirrhosis and further increased by platelet transfusion

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## Summary

**Background:** Thrombocytopenia and circulating dysfunctional immune cells are commonly observed in patients with cirrhosis. Platelets may form complexes with neutrophils, monocytes and T cells modulating their function. We recently reported increased frequencies of platelet-complexed neutrophils in cirrhosis with evidence of neutrophil activation upon contact with healthy platelets *in vitro*. Whether this occurs *in vivo* following platelet transfusion and contributes to systemic inflammation and endothelial activation is unknown.

**Aims:** To characterise platelet-leucocyte aggregation in cirrhosis and to determine whether elective platelet transfusion results in perturbations associated with changes in markers of haemostasis, inflammation or endothelial activation.

**Methods:** We collected blood from cirrhotics ( $n = 19$ ) before and following elective platelet transfusion. We measured platelet-leucocyte aggregation, activation and function, and markers of platelet activation, systemic inflammation and endothelial activation by flow cytometry. Haemostasis was assessed by thromboelastometry and plasma haemostatic proteins.

**Results:** We observed a 2.5-fold increase in platelet-complexed neutrophils in patients with cirrhosis compared with healthy subjects and twofold more platelets attached per monocyte and T cell. All platelet-complexed leucocytes expressed higher levels of activation markers and platelet-complexed neutrophils had higher resting oxidative burst and phagocytic capacity than their nonplatelet-complexed counterparts ( $P < 0.001$ ); most pronounced in patients with cirrhosis. Paradoxically, platelet-complexed leucocyte frequency decreased with increasing MELD score. Platelet transfusion increased soluble CD40 ligand (platelet activation marker), the frequency of platelet-complexed monocytes ( $P < 0.05$ ) and improved haemostatic status.

**Conclusion:** Cirrhotic patients have activated circulating platelet-complexed leucocytes with increased platelet-monocyte aggregation following elective platelet transfusion. Elective platelet transfusion might therefore exacerbate immune dysfunction in cirrhosis.

## 1 | INTRODUCTION

Functional alterations in circulating immune cells are well recognised in patients with cirrhosis and predict the development of infection, organ dysfunction and thus mortality. The immunological disturbance is complex and encompasses both hyper-activation of immune cells, which can cause injury, and functional immune paralysis impairing the ability to fight infection.<sup>1–5</sup> Thrombocytopaenia is also a well-established feature of cirrhosis with portal hypertension and circulating platelets have been reported to be in a pre-activated state.<sup>6</sup> Platelets are key in thrombosis and haemostasis, but have functions beyond this. Notably, platelets also hold vital immunomodulatory properties and may therefore be involved in the functional leucocyte defects observed in cirrhosis.

Upon activation, platelets can become attached to leucocytes leading to the formation of platelet-leucocyte complexes in circulation.<sup>7</sup> Platelet-leucocyte aggregation is enhanced by the presence of endotoxin and pre-activation of the platelets both of which are observed in cirrhosis.<sup>8,9</sup> Complex formation with platelets enhances oxidative burst and degranulation of neutrophils and monocytes and promotes their tissue extravasation.<sup>8,10</sup> The latter is enforced by direct activation of the endothelium by activated platelets.<sup>11–14</sup> When platelets interact with lymphocytes, it facilitates their entry into lymph nodes.<sup>15</sup> Platelets may thus perpetuate the leucocyte priming that is already present in cirrhosis.

There is a paucity of data on platelet-mediated immune regulation in cirrhosis. In a recently published *ex vivo* study, platelets isolated from patients with cirrhosis had a decreased capability to temper neutrophil priming and production of reactive oxygen species (ROS).<sup>16</sup> However, paradoxically, the addition of platelets isolated from healthy individuals to cirrhotic neutrophils increased the formation of platelet-complexed neutrophils (PCN) and neutrophil adhesion receptor, CD11b, expression.<sup>16</sup> Such primed neutrophils are suspected to be responsible for end-organ damage in sepsis probably owing to augmented systemic inflammation and endothelial activation.<sup>16,17</sup> High frequencies of platelet-leucocyte aggregates have been associated with a pro-thrombotic state and are thought to be implicated in several cardiovascular diseases.<sup>18</sup> This is of particular concern as patients with cirrhosis and thrombocytopenia frequently undergo platelet transfusions prior to invasive procedures.

We therefore investigated, firstly, whether there is altered platelet aggregation with neutrophils, monocytes and T cells and consequent functional changes in these cells in patients with cirrhosis. Secondly, whether a transfusion with healthy platelets given to a patient with cirrhosis electively prior to an invasive procedure impacts in any way on these measures. We hypothesised that platelet-leucocyte aggregation is augmented in cirrhosis and that this increases leucocyte activation, oxidative stress, systemic inflammation and endothelial activation. Furthermore, we hypothesised that elective platelet transfusion will exacerbate these events.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design and population

In this prospective cohort study, we consecutively recruited 19 patients with cirrhosis from King's College Hospital Liver Unit between March and August 2015 scheduled to receive elective platelet transfusion prior to invasive procedures. Platelets were transfused prior to procedures when the platelet count was below  $80 \times 10^9/L$  according to the local guideline. Blood samples were taken prior to the administration of platelets and repeated between 1 and 3 (mean  $\pm$  SD;  $1.7 \pm 0.8$ ) hours after commencing transfusion. We aimed to leave as long as possible from transfusion start to sampling, but with sampling occurring before the invasive procedure and before transfusion of additional blood products. All post-transfusion blood samples were taken prior to the invasive procedure except for one case that was taken after variceal band ligation. Platelet transfusions were in all but two cases ABO matched, and consisted of either pooled platelets or apheresed platelets. Patients had between 1 and 2 pools of platelets depending on their platelet count. Blood was taken from healthy subjects ( $n = 9$ ) as controls.

### 2.2 | Inclusion and exclusion criteria

Patients with ultrasonic and/or histological verification of cirrhosis of any aetiology, between the ages of 18 and 75, and scheduled for elective platelet transfusion were included. Healthy subjects with no history of liver and thrombotic disease were included as controls. Findings characteristic of cirrhosis on ultrasound included a coarse, nodular heterogeneous parenchyma with or without evidence of the development of portal hypertension with splenomegaly, sluggish portal blood flow and the development of ascites. Patients were excluded if they had evidence of an acute infection, had undergone liver transplantation or had hepatocellular carcinoma outside the Milan criteria.<sup>19</sup> Patients were also excluded if they had received any platelet transfusion in the previous 2 weeks or had been treated with any anti-platelet therapies, anti-coagulants or immunomodulatory drugs. Likewise, none of the healthy subjects recruited had acute infection or had received any of the above drugs. Patients were excluded after inclusion if plasma was given before or alongside platelet transfusion or if other drugs affecting leucocyte or platelet function were given within the study time frame.

### 2.3 | Consent and data collection

The study was granted ethical approval by the North East London Research Ethics Committee [Ref 08/H0702/52] and was conducted in accordance with the Declaration of Helsinki. Written, informed consent was obtained from all study participants prior to study inclusion. Clinical and biochemical data were collected and model of end-stage liver disease (MELD) and Child-Pugh scores were calculated.

## 2.4 | Blood sampling

Venous blood was collected in citrated and EDTA tubes. A tourniquet was used as part of the blood collection procedure, but stasis was reduced to the absolute minimum. Samples were transported at room temperature and thromboelastometry and flow cytometry assays were performed immediately after blood was drawn. Plasma was obtained by centrifugation of whole blood and stored at  $-80^{\circ}\text{C}$  for later cytometric bead array analyses and analyses of haemostatic proteins.

## 2.5 | Quantification of platelet-complexed leucocytes and leucocyte phenotyping

A whole blood surface staining was performed with optimised amounts of fluorochrome-conjugated antibodies; CD41a-PE, CD41a-APC, CD16-PE, CD11b-APC-CY7, CD14-PerCP-Cy5.5, CD4-APC-H7, CD8-APC, CD25-Per-CY7, CD69-FITC, CD45RO-PerCP-cy5.5, (Becton Dickinson (BD), UK). Erythrocyte lysis was performed after surface staining with lysis solution (BD, UK) to avoid activation of the platelets by erythrocyte lysis. Samples were analysed using a FACS Canto II analyser (BD, San Jose, USA). Platelet-leucocyte aggregates were identified by co-expression of the platelet marker CD41a (platelet glycoprotein alpha-IIb) with CD16<sup>+</sup> granulocytes; PCN, with CD14<sup>+</sup>CD16<sup>-</sup> (classical monocytes), CD14<sup>+</sup>CD16<sup>+</sup> (intermediate monocytes) and CD14<sup>lo</sup>CD16<sup>+</sup> (non-classical monocytes); platelet-complexed monocytes (PCM) and CD4<sup>+</sup> or CD8<sup>+</sup> T cells; platelet-complexed T cells (PCT). Median fluorescence intensity (MFI) of the adhesion receptor CD11b was measured on neutrophils and monocytes as a marker of activation and for T cells the MFI of the IL-2 receptor, CD25, and CD69 were determined. Unstained samples and fluorescence minus ones (FMOs) were included in all flow cytometry experiments as controls.

## 2.6 | Leucocyte function tests

Neutrophil oxidative burst and phagocytosis were measured as previously described and degranulation status was measured on both neutrophils and monocytes.<sup>20</sup>

### 2.6.1 | Oxidative burst

Oxidative burst was quantified using the PhagoBurst kit (Orpegen Pharma, Heidelberg, Germany), which detects rhodamine oxidation as a measure of ROS production in unstimulated neutrophils (resting burst) and after stimulation with formyl-Met-Leu-Phe (fMLP) (low burst), phorbol 12-myristate 13-acetate (PMA) (high burst) or opsonised *E. coli* (phagoburst) following the manufacturer's instructions.

### 2.6.2 | Phagocytic activity and capacity

Phagocytosis was measured with FITC-labelled *E. coli* using the PhagoTest kit (Orpegen Pharma, Heidelberg, Germany) as per manufacturer's instructions.

In both assays, cells were stained with CD16-PE and CD41a-APC for 30 minutes in the dark to identify PCN. Samples were analysed immediately after preparation. Neutrophil ROS production was reported as the percentage of rhodamine-positive PCN and as MFI. Neutrophil phagocytic activity was reported as the percentage of *E. coli*-positive PCN and neutrophil phagocytic capacity as the median number of *E. coli* present within each neutrophil (MFI).

### 2.6.3 | Degranulation

Neutrophil and monocyte degranulation markers were measured both in the extracellular and intracellular compartments in unstimulated and *E. coli*-stimulated cells. One hundred microlitres of whole blood were incubated with 380  $\mu\text{L}$  RMPI and 20  $\mu\text{L}$  PBS or opsonised *E. coli* at  $37^{\circ}\text{C}$  for 20 minutes. After washing, half of the tubes were stained for MPO, CD66b, CD11b, CD107a, CD63 and CD16 (BD, UK) and the other half for CD16 only. Following 30 minutes of incubation, the cells were washed and lysed and intracellular tubes were permeabilised with 100  $\mu\text{L}$  of cytofix/cytoperm solution (BD, UK) and stained with the above-mentioned degranulation markers. Expression of the degranulation markers was reported as MFI.

## 2.7 | Cytometric bead array

The markers of platelet activation (sP-selectin and sCD40L), the pro-inflammatory cytokine monocyte chemoattractant protein-1 (MCP-1) and the endothelial activation markers (sICAM-1, sVCAM-1 and sP-selectin) were quantified using cytometric bead array (BD, UK) from the previously stored plasmas.

## 2.8 | Thromboelastometry

Thromboelastometry was performed on citrated whole blood using Rotem and the assay panel included INTEM, EXTEM, FIBTEM and APTEM. INTEM and EXTEM are haemostasis-screening tests with by testing the activation of the intrinsic (contact phase) or extrinsic (tissue factor) pathways respectively. In FIBTEM, cytochalasin-D was used to inhibit platelets thus isolating the function of fibrin in clot formation, and in APTEM, fibrinolysis was inhibited by aprotinin enabling the detection of hyperfibrinolysis. Maximal runtime of the samples was 60 minutes.

## 2.9 | Haemostatic assays

Platelet counts were assessed in whole blood using routine diagnostic methods. In plasma samples, markers of primary haemostasis (platelet count, von Willebrand factor [VWF], ADAMTS13), coagulation (factor VIII, prothrombin time [PT], international normalised ratio [INR], activated partial thromboplastin time [APTT], antithrombin and fibrinogen) and thrombin generation (F1 + 2, thrombin-anti-thrombin complexes [TAT]) were quantified as previously described.<sup>21,22</sup> Plasma fibrinolytic capacity was measured utilising a plasma-based clot lysis time assay, also described previously.<sup>21,22</sup> Plasma thrombin generating

potential was measured in vitro in the presence and absence of thrombomodulin by calibrated automated thrombography.

## 2.10 | Statistics

Statistical analyses were performed using nonparametric tests. To assess differences between groups (cirrhosis patients' vs healthy subjects) we employed Wilcoxon rank-sum test. Wilcoxon signed-rank test was used to compare paired data (before and following platelet transfusion) and Spearman's rho for correlation analyses. Results are expressed as median and interquartile range (25%; 75%), and a  $P < 0.05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | Patient baseline characteristics

We recruited patients with cirrhosis of any aetiology; alcohol ( $n = 8$ ), cleared hepatitis C virus infection ( $n = 5$ ), nonalcoholic fatty liver disease/steatohepatitis ( $n = 2$ ), Wilson's disease ( $n = 1$ ), primary biliary cholangitis ( $n = 1$ ), primary sclerosing cholangitis ( $n = 1$ ), cryptogenic cirrhosis ( $n = 1$ ). Patient clinical and biochemical characteristics at inclusion are presented in Table 1. Three of the

**TABLE 1** Baseline characteristics of the cirrhosis patients prior to platelet transfusion

Characteristics	Cirrhosis
Gender: F/M	6/13
Age (y)	53 (46;66)
Aspartate transaminase (10-50 U/L)	48.5 (34;66)
Bilirubin (0-21 $\mu\text{mol/L}$ )	28 (21;85)
Alkaline phosphatase (35-129 U/L)	144 (94;180)
Gamma glutamyl transferase (5-55 U/L)	72 (27;137)
Albumin (40-52 g/L)	30 (24;38)
Sodium (135-145 mmol/L)	137 (132;140)
Creatinine (45-104 $\mu\text{mol/L}$ )	70 (51;94)
INR (2.0-3.3)	1.62 (1.25;1.84)
Haemoglobin (g/L)	109 (98;125)
Platelets ( $\times 10^9/\text{L}$ )	51 (33;61)
White blood cell count ( $\times 10^9/\text{L}$ )	3.23 (3.23;2.03)
Neutrophils ( $\times 10^9/\text{L}$ )	2.0 (1.14;3.84)
Monocytes ( $\times 10^9/\text{L}$ )	0.27 (0.17;0.50)
Lymphocytes ( $\times 10^9/\text{L}$ )	0.72 (0.51;1.30)
MELD score	16 (10;23)
Child-Pugh score	9 (6;10)
(A/B/C)	(5/8/6)
Temperature ( $^{\circ}\text{C}$ )	36.6 (36.3;36.8)

Values are presented as median (IQR).

INR, international normalised ratio; MELD, model of end-stage liver disease; IQR, interquartile range.

patients were actively drinking above the recommended limits and 8 patients were receiving beta-blockers. These patients did not differ from the rest of the cohort on our outcome measures. Using thromboelastometry, we demonstrated significantly impaired haemostasis in this cohort of cirrhosis patients compared with controls (Table 2). The recruited patients underwent the following procedures: variceal banding ( $n = 6$ ), dental procedures ( $n = 4$ ), large volume paracentesis ( $n = 3$ ), tracheostomy ( $n = 2$ ), liver biopsy ( $n = 1$ ) and radiofrequency ablation ( $n = 1$ ). One patient was excluded from the pre-post-comparisons as granulocyte colony-stimulating factor (G-CSF) was given alongside platelet transfusion and for 1 patient platelet transfusion was cancelled following inclusion. None of the patients experienced haemorrhagic or thrombotic procedure-related complications.

### 3.2 | Elevated frequencies of PCN and platelet-complexing with monocytes and T cells in cirrhosis

In patients with cirrhosis, the frequency of PCN was 2.5-fold higher than in healthy subjects (Figure 1A) and a similar tendency was evident in the subset of intermediate monocytes (Figure 1B). The frequency of platelet complexes was not different in the other leucocyte subsets investigated (Figure 1C-F). However, a greater number of platelets were attached per individual monocyte in the population of both classical and intermediate monocytes as well as in T cells in patients with cirrhosis (Figure 1G). A similar tendency was seen in PCN.

### 3.3 | Platelet-complexed leucocytes are more activated than noncomplexed leucocytes especially in cirrhosis

Neutrophils and monocytes that form complexes with platelets were more activated as measured by the expression of CD16 and CD11b than noncomplexed neutrophils and monocytes in cirrhosis (neutrophil CD16;  $P = 0.0001$ ) (Figure 2A). Also for T cells, the expression of CD69 and CD25 was higher on platelet-complexed than noncomplexed T cells (Figure 2B-C). These differences between platelet-complexed and noncomplexed neutrophils, monocytes and T cells were also found in healthy subjects ( $P < 0.05$ ) [data not shown]. However, this difference in expression of activation markers between complexed and noncomplexed leucocytes was greater in patients with cirrhosis than in healthy subjects in both neutrophils (CD16,  $P = 0.0008$ ) and monocytes (Figure 2D). The same was evident for CD25 expression in CD4<sup>+</sup> T cells, but not the case for CD69 or for CD8<sup>+</sup> T cells (Figure 2E).

### 3.4 | Increased resting and stimulated burst and phagocytic capacity in PCN

Platelet-complexed neutrophils had a higher resting oxidative burst ( $P < 0.001$ ) than neutrophils not in complex with platelets (Figure 3A). Additionally, PCN responded with a higher oxidative burst

**TABLE 2** Thromboelastometric improvement in haemostasis following platelet transfusion in patients with cirrhosis

		Healthy		Healthy vs cirrhosis pre-plt tx P value <sup>a</sup>	Cirrhosis pre-plt tx		Cirrhosis post-plt tx		Cirrhosis pre-plt tx vs post-plt tx P value <sup>b</sup>
		Median	(IQR)		Median	(IQR)	Median	(IQR)	
IN	CT	201	(178;217)	0.41	205	(196;222)	199.5	(180;218)	0.085
IN	CFT	80	(70;97)	0.0002*	194	(154;247)	174.4	(142;219)	0.03*
IN	Alpha	75	(72;76)	0.0004*	64	(57;70)	66.5	(61;70)	0.0002*
IN	MCF	60	(57;62)	0.0001*	43	(37;49)	47.5	(41;51)	0.002*
IN	A15	57	(54;60)	0.0001*	37	(32;44)	42	(35;46)	0.005*
IN	ML	6	(4.5;11)	0.65	8	(4;12)	6	(4;8)	0.0002*
EX	CT	67	(65;69)	0.31	76	(61;84)	68	(60;76)	0.01*
EX	CFT	93.5	(86.5;113)	0.0002*	187	(150;271)	177.5	(143;212)	0.006*
EX	Alpha	71	(67.5;72.5)	0.01*	63	(55;69)	65	(57;71)	0.0002*
EX	MCF	63	(59.5;65.5)	0.0001*	45	(40;51)	48.5	(43;53)	0.001*
EX	A15	59	(54;60.5)	0.0001*	40	(34;44)	41	(38;47)	0.002*
EX	ML	6.5	(4;11)	0.46	9	(6;13)	7	(4;8)	0.0002*
AP	CT	65	(60;73)	0.59	68	(59;76)	68	(61;77)	0.26
AP	CFT	98	(90;116)	0.0003*	200	(162;251)	173.5	(138;220)	0.004*
AP	Alpha	71	(67;72)	0.12	66	(53;71)	64.5	(56;70)	0.0002*
AP	MCF	62	(60;64)	0.0006*	48	(40;52)	48.5	(44;53)	0.05
AP	A15	58	(54;59)	0.0002*	40	(34;44)	41	(37;47)	0.009*
AP	ML	5.5	(4;11)	0.51	11	(2;13)	7	(3;03)	0.0002*
FI	CT	67	(60.5;70.5)	0.92	66	(59;79)	65	(60;74)	0.13
FI	MCF	11	(10.5;12)	0.36	12	(10;16)	14.5	(8;17)	0.45
FI	A15	11	(10.5;11.5)	0.63	11	(9;15)	12.5	(8;15)	0.16
FI	ML	2.5	(0.5;4)	0.77	2	(1;7)	3.5	(1;9)	0.01*

Thromboelastometry was performed on a Rotem instrument on citrated whole blood in healthy subjects and patients with cirrhosis before and at 1-3 hours following an elective platelet transfusion and the results compared. Values are presented as median (IQR).

Plt tx, platelet transfusion; INTEM, (IN) haemostasis activated by contact; EXTEM, (EX) haemostasis activated by tissue factor; APTTEM, (AP) fibrinolysis inhibited and FIBTEM, (FI) platelets inhibited assays were performed; CT, clotting time; CFT, clot formation time; alpha, speed of clot formation; MCF, maximum clot firmness; A15, clot firmness at 15 minutes; ML, maximum lysis.

<sup>a</sup>ranksum.

<sup>b</sup>signrank.

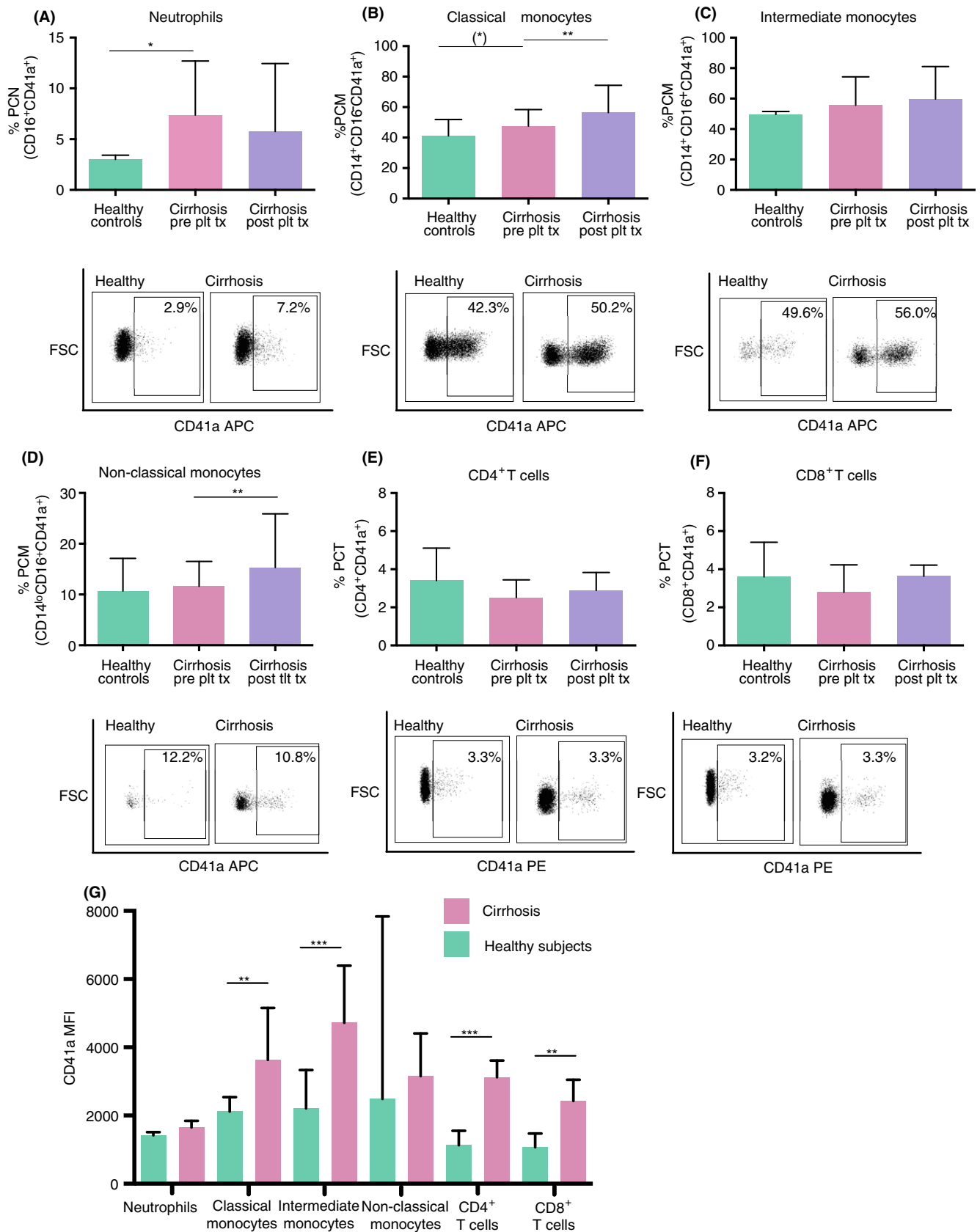
\* $P < 0.05$ .

when exposed to fMLP than the noncomplexed neutrophils (Figure 3B). This was not found for the PMA (Figure 3C) or *E. coli*-induced burst. Similar differences between complexed and noncomplexed cells were found in healthy subjects [data not shown]. The differences in burst when comparing platelet-complexed with noncomplexed neutrophils were greatest in patients with cirrhosis for resting burst and fMLP-stimulated burst and indifferent for PMA (Figure 3D-F) and *E. coli*-induced burst. PCN had a higher phagocytic capacity than noncomplexed neutrophils in cirrhosis (Figure 3G) and in healthy subjects ( $P = 0.03$ ), however, the difference in phagocytic capacity between complexed and noncomplexed neutrophils was equal between the groups (Figure 3H). We also investigated neutrophil and monocyte degranulation, but found no correlations between PCN or PCM frequency or activation and baseline markers of degranulation [data not shown].

### 3.5 | PCN, PCM, platelet and endothelial activation is reduced in those with advanced cirrhosis consistent with immune exhaustion

Paradoxically, the activation state of PCN was inversely related to MELD score ( $r = -.45$ ,  $P = 0.05$ ) and similarly for nonclassical PCM ( $r = -.52$ ,  $P = 0.03$ ) consistent with those with end-stage liver disease having evidence of immune exhaustion. The frequency and activation state of platelet-leucocyte aggregates were not related to platelet count. The platelet activation markers, sCD40L and P-selectin, were also inversely related with Child-Pugh score ( $r = -.46$ ,  $P < 0.05$ ) and ( $r = -.42$ ,  $P = 0.076$ ). Furthermore, the endothelial activation marker sVCAM-1 was inversely related with both MELD ( $r = -.74$ ,  $P < 0.01$ ) and Child-Pugh scores ( $r = -.82$ ,  $P < 0.001$ ) [data not shown].



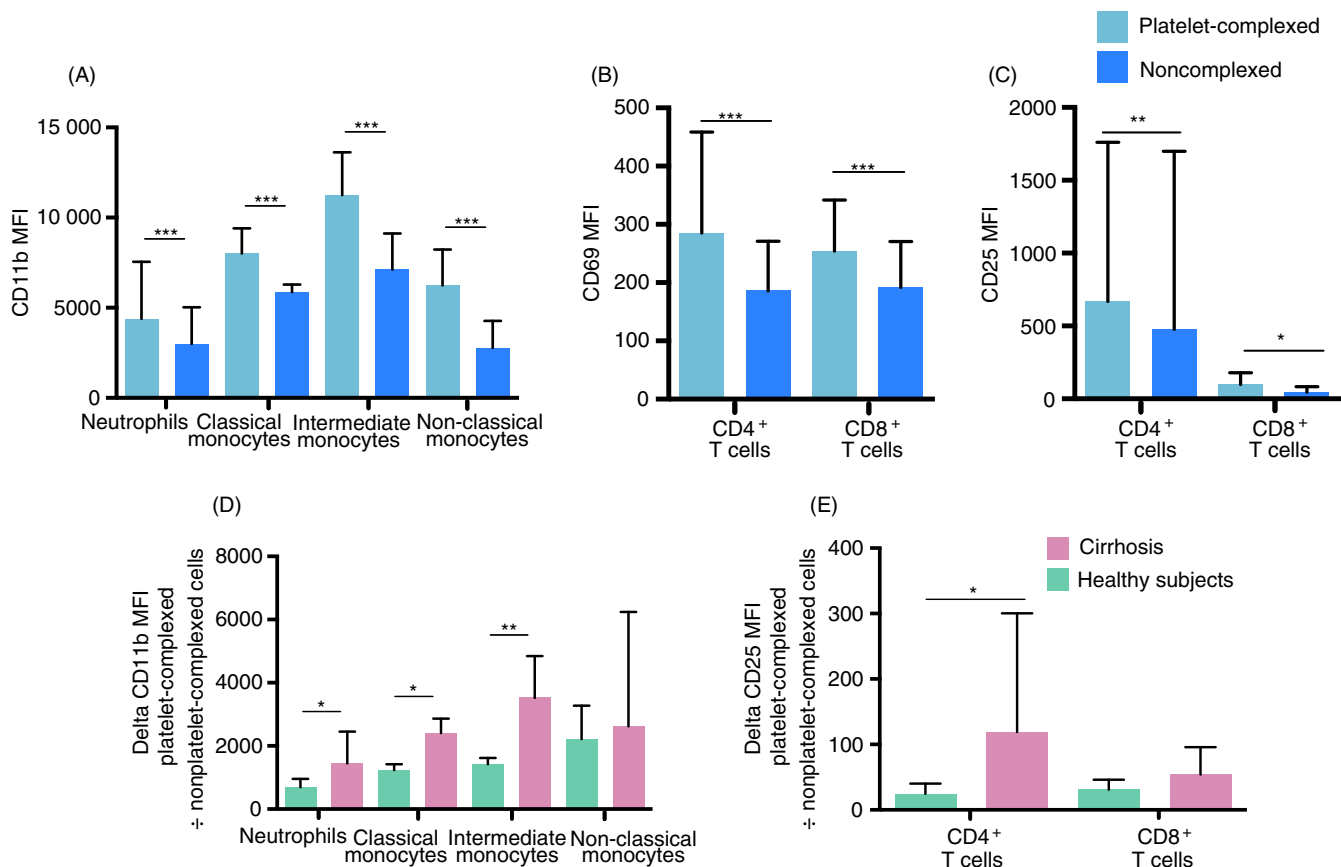


**FIGURE 1** Platelet complexing with leucocytes is augmented in cirrhosis. Platelet-complexed neutrophils, monocytes and T cells were quantified by flow cytometry as the co-expression of the platelet glycoprotein IIb, CD41a, with leucocyte population markers. (A-F), The frequency of these complexes was compared between patients with cirrhosis and healthy subjects (ranksum) and patients with cirrhosis before (pre-plt tx) and following (post) platelet transfusion (signrank). Representative dotplots shown below figures. Unstained samples and fluorescence minus ones (FMOs) were included in all flow cytometry experiments as controls. (G), Median fluorescence intensity (MFI) of the platelet marker CD41a on the different leucocyte populations. (\*)  $P = 0.1$  \* $P < 0.05$  \*\* $P < 0.01$ , \*\*\* $P < 0.001$

### 3.6 | Changes in platelet-leucocyte aggregation induced by platelet transfusion are reflected in changes in leucocyte activation

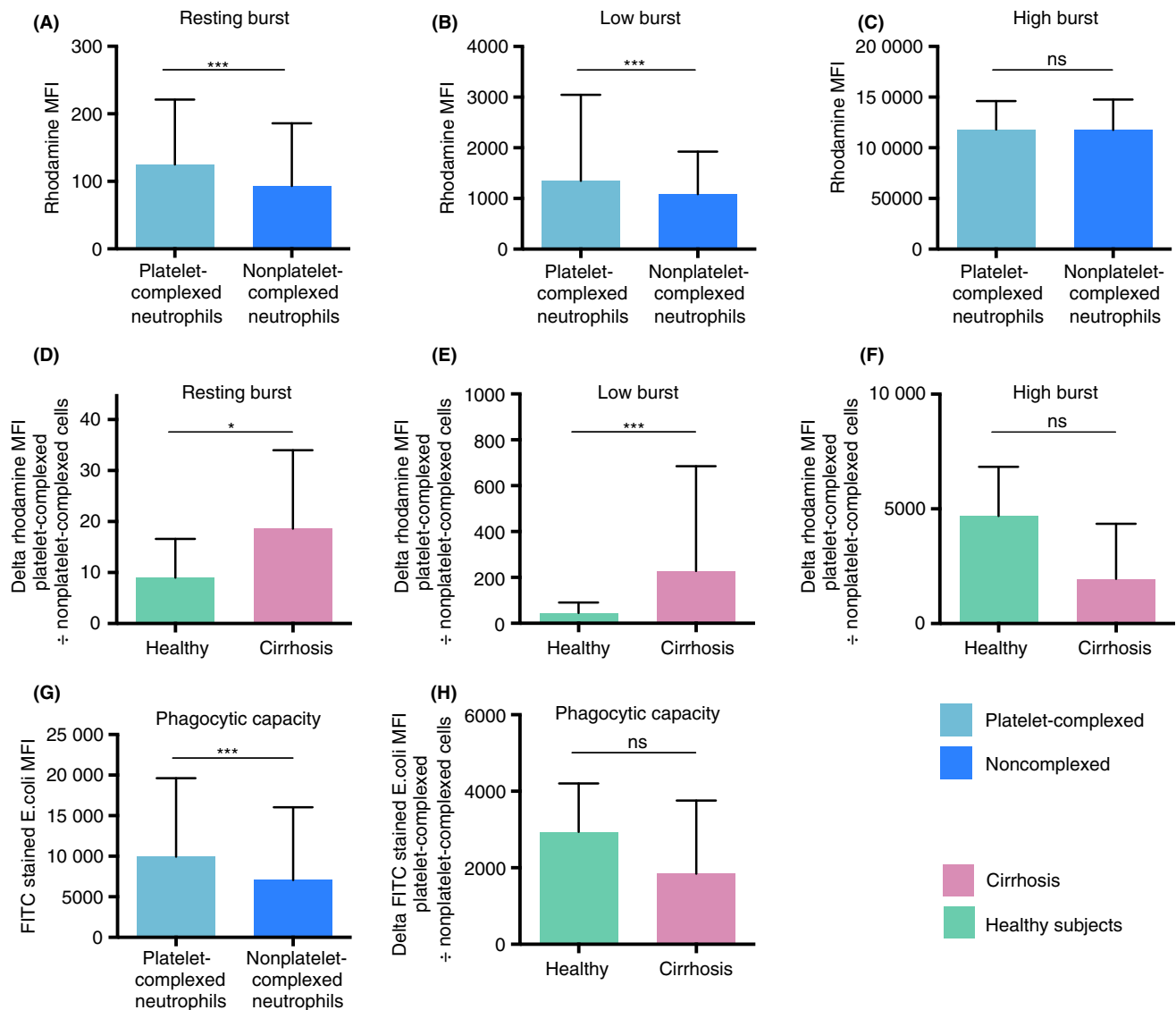
The frequency of PCN did not significantly differ following platelet transfusion in this cohort of cirrhosis patients (Figure 1A). When looking at the individual patients, we found that 9 of the 17 patients responded to transfusion by an increase in PCN and in 8 of the patients the percentage decreased. The change in PCN frequency prior, and following platelet transfusion, however, correlated with the change in neutrophil CD11b ( $r = .80$ ,  $P < 0.01$ ) and CD16

( $r = .52$ ,  $P < 0.01$ ) expression. For monocytes, the percentage of PCM increased following platelet transfusion in the classical population by 20% and in the non-classical monocytes by 35% (Figure 1B, D). Like the neutrophils, we observed a positive correlation between the changes in the percentage of PCM and the CD11b expression in the classical monocyte population ( $r = .61$ ,  $P < 0.05$ ) and a similar tendency in the population of non-classical monocytes ( $r = .41$ ,  $P = 0.1$ ). The percentage of platelet-complexed T cells did not change following platelet transfusion (Figure 1E, F). As for the other leucocytes, changes in the percentage of platelet-complexed T cells were reflected in activation changes [data not shown].



**FIGURE 2** Platelet-complexed leucocytes are more activated than noncomplexed leucocytes especially in cirrhosis. (A), Expression of the adhesion receptor CD11b was measured by flow cytometry on platelet-complexed neutrophils and monocytes and compared with the expression on nonplatelet-complexed neutrophils and monocytes. The expression of CD69 (B), and CD25 (C), was similarly measured on platelet-complexed CD4<sup>+</sup> and CD8<sup>+</sup> T cells and compared with noncomplexed T cells. (D), The difference in CD11b expression between platelet-complexed and noncomplexed neutrophils and monocytes in cirrhotic patients was compared with healthy subjects. (E), The difference in CD25 expression on platelet-complexed and noncomplexed T cells was compared between the groups. Expression is reported as median fluorescence intensity (MFI). Ranksum/signrank, \* $P < 0.05$  \*\* $P < 0.01$ , \*\*\* $P < 0.001$





**FIGURE 3** Platelet-complexed neutrophils have higher resting burst, low burst and phagocytic capacity than noncomplexed neutrophils especially in cirrhosis. Citrated whole blood was incubated without stimulation (resting burst) or with formyl-Met-Leu-Phe (fMLP) (low burst), phorbol 12-myristate 13-acetate (PMA) (high burst) and the production of reactive oxygen species detected by the conversion of dihydro-rhodamine to rhodamine. Phagocytosis was measured by incubation with opsonised FITC-labelled *E. coli* bacteria (phagocytic capacity). Platelet-complexed neutrophils (PCN) were identified by the expression of CD16 and CD41a. Burst/phagocytic capacity is presented as median fluorescence intensity (MFI). (A-C), Burst in PCN compared with and nonPCN. (D-F), The difference in burst between PCN and nonPCN is compared between healthy subjects and patients with cirrhosis. (G), Phagocytic capacity in PCN compared with nonPCN. (H), The difference in phagocytic capacity between PCN and non-PCN is compared between healthy subjects and patients with cirrhosis. Ranksum and signrank, ns: not significant, \* $P < 0.05$ , \*\*\* $P < 0.001$

### 3.7 | Plasma sCD40L level increase after platelet transfusion

The plasma level of sCD40L increased following platelet transfusion ( $P < 0.05$ ) but none of the other markers of systemic inflammation or endothelial activation changed (Figure 4).

### 3.8 | Leucocyte function is not affected by platelet transfusion

We investigated whether platelet transfusion induced functional changes in the leucocytes. However, neither neutrophil oxidative

burst nor phagocytosis was significantly different following platelet transfusion (Table S1) and markers of degranulation expressed on neutrophils and monocytes did not change (Table S2). T-cell maturation as measured by CD45RO was also unchanged.

### 3.9 | Platelet transfusion improves haemostatic status

Table 2 demonstrates an improvement in haemostatic status following platelet transfusion as measured by thromboelastometry; time to clot development was reduced (CT), the onset of clot formation

occurred more rapidly (CFT, Alpha) and a greater clot firmness was obtained (MCF). Additionally, clot lysis was decreased (ML). In the FIBTEM assays, where platelets are inhibited by cytochalasin-D, these effects were not seen and fibrinolysis was increased. In addition, we measured haemostasis parameters in plasma and detected a highly significant decrease in factor VIII and an increase in thrombin-antithrombin complexes following platelet transfusion (Figure 5). No clear changes in other haemostatic parameters assessed were observed (Figure S1).

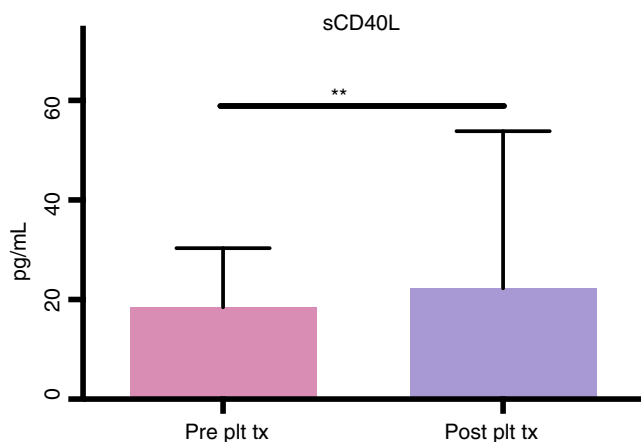
## 4 | DISCUSSION

In this study, we sought to evaluate the extent of platelet aggregation with neutrophils, monocytes and T cells in patients with cirrhosis and observed augmented platelet-complex formation in all investigated leucocyte subsets. Platelet aggregation with neutrophils and T cells has to our knowledge not previously been investigated in

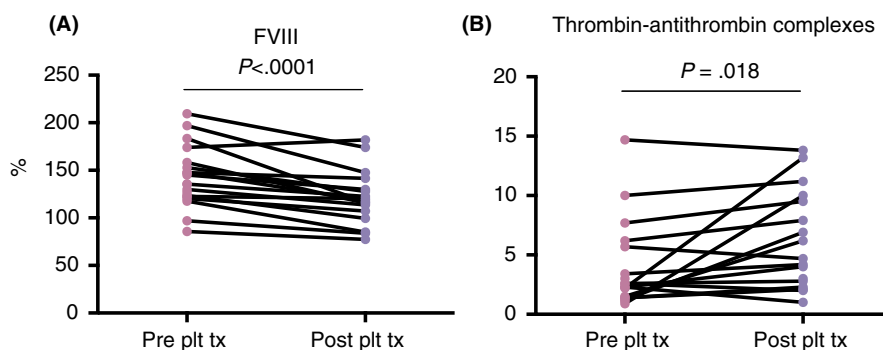
patients with cirrhosis. Studies assessing the frequencies of PCM in cirrhosis are conflicting.<sup>9,23,24</sup> The diverging data may be explained by different disease severities investigated and particularly the experimental circumstances; choice of anti-coagulant, platelet marker and time to analyses. As platelet-leucocyte aggregation is greatly affected by these choices, it is vital to treat control and patient samples similarly.<sup>25</sup> The studies reporting elevated PCM frequencies in cirrhosis detected much lower frequencies of complex formation in their healthy control cohorts than we did, while other reports are in agreement with our values.<sup>9,24,26</sup> A study evaluating platelet aggregation in monocyte subsets detected preferential platelet complexing with CD16<sup>+</sup> monocytes in accordance with our findings.<sup>23</sup>

Studies support that platelet-complexed leucocytes are a phenotypically and functionally distinct compartment of leucocytes that are primed for tissue extravasation and microbial killing.<sup>26,27</sup> However, this study is the first to show an augmentation of this platelet-induced leucocyte activation in cirrhosis. Thrombocytopenia may in itself act as a stimulant as a high platelet-to-neutrophil ratio has been shown to aid ROS production.<sup>28</sup> In addition, ongoing platelet activation in cirrhosis may also contribute to augmented platelet-leucocyte complex formation.<sup>29,30</sup> The presence of platelet activation in patients with cirrhosis has however been debated as the elevated soluble platelet activation markers are cleared by the liver and could solely reflect hepatic dysfunction.<sup>31</sup> The findings which support the presence of platelet activation in cirrhosis are increased platelet aggregation, along with increased expression of surface markers measured by flow cytometry, but no consensus has been reached.<sup>9</sup> Due to the methodological difficulties in accurately characterising the activation status of circulating platelets, assessing the frequencies of PCM has been proposed as a sensitive and reliable indicator of platelet activation.<sup>32</sup> Based upon our findings and on the conflicting reports of PCM frequencies in cirrhosis, we suggest PCN frequency to be a more sensitive index of platelet activation in patients with cirrhosis.

In agreement with previous studies, we observed lower frequencies of PCN and PCM with increasing disease severity, which was not explained by lowered platelet counts.<sup>23</sup> A similar pattern of enhanced complex formation occurs during the systemic pro-



**FIGURE 4** Plasma soluble CD40 ligand (sCD40L) is increased after platelet transfusion in patients with cirrhosis. We employed a cytometric bead array to measure sCD40L in EDTA plasma. Levels were compared before and following platelet transfusion (plt tx) in patients with cirrhosis. Signrank, median, interquartile range, \*\* $P < 0.01$



**FIGURE 5** Platelet transfusion decreases factor VIII and increases thrombin-antithrombin complexes. The plasma levels of (A), factor VIII and (B), thrombin-antithrombin complexes were measured in plasma before and following an elective platelet transfusion (plt tx) in patients with cirrhosis and the levels compared (signrank)

inflammatory surge in sepsis and with a retraction in platelet-leucocyte aggregation as sepsis progresses to septic shock.<sup>33</sup> This may reflect immune paresis, which frequently follows immune hyper-activation, and which may be related to deposition of platelet-leucocyte complexes in the organs as observed in experimental models of sepsis.<sup>34</sup>

We repeated our analyses following an elective platelet transfusion to assess the effects on the platelet-leucocyte interplay. We verified utilising thromboelastometry and plasma-based haemostasis tests, that elective transfusion of 1 or 2 pools of platelets impacts on haemostatic status in these patients. Whether these changes are clinically meaningful, however, remain to be seen. Plasma levels of sCD40L increased following platelet transfusion, which is indicative of platelet activation. However, it has been well-established that the handling and storing of platelets results in slight platelet activation and increases sCD40L in the transfusate and this can increase plasma sCD40L after transfusion.<sup>35</sup> Possibly, the cirrhotic platelets also contribute to this plasma increase, as sCD40L acts as a cytokine and can activate other platelets.<sup>36,37</sup>

Consistent with a higher number of circulating platelets following transfusion and their increased activation, the frequency of PCM increased. This increase was not evident in neutrophils and T cells in our cohort, but we observed that some patients responded to platelet transfusion with an increase, and others with a decrease, in platelet-leucocyte aggregation. These differential responses are likely to be a result of the heterogeneity of our cohort, but our limited sample size prevents us from identifying the responsible factor. Nevertheless, the fact that changes in platelet-leucocyte aggregation in response to platelet transfusion correlated with increased expression of activation markers on neutrophils, monocytes and T cells suggest a potential priming effect of platelet transfusion. That platelets stored for transfusion are apt to aggregate with leucocytes and cause their activation has previously been demonstrated *in vitro*.<sup>38</sup> We cannot say from this study whether this response is unique to cirrhosis patients, but we speculate that this response may at least be more pronounced in these patients who at baseline have enhanced activation of their platelet-complexed leucocytes.

The pathophysiological consequences of elevated platelet-leucocyte aggregation in patients with cirrhosis and its enhancement by platelet transfusion in certain individuals may be of both haemostatic and immunological nature. Platelet-leucocyte aggregates are regarded as pro-thrombogenic, which mechanistically is explained by increased tissue factor production by the involved leucocytes accompanied by enhanced fibrin deposition.<sup>39–41</sup> This may add to the already increased risk of venous thrombosis in these patients.<sup>42</sup> Indeed, the increase in thrombin-antithrombin complexes following platelet transfusion suggests a prohaemostatic effect of donor platelets. Immunologically, further priming of leucocytes within the aggregates augments any pre-existing cirrhosis-induced hyper-activation and could therefore potentiate systemic inflammation and endothelial activation. Indeed, in sepsis-induced multiple organ failure, activated neutrophils have been proposed responsible for inducing or augmenting end-organ injury.<sup>17</sup> Platelet CD40L may also, besides

any activating effects on leucocytes, act on endothelial cells to produce chemokines and up-regulate adhesion receptors that promote tissue extravasation of leucocytes.<sup>43</sup> However, in our study we did not see any changes in systemic inflammation or endothelial activation markers after platelet transfusion. Ideally, we would have liked to measure these markers as well as leucocyte function at 12 and 24 hours following platelet transfusion but as transfusions were performed prior to invasive procedures introducing strong confounding factors such as sedatives, stress and mechanical injury, we chose the latest time point before procedure to isolate the effects of the transfusion. Prior to initiation of the study, we performed a time course experiment on 2 patients with sampling at 1, 3 and 5 hours after transfusion, which showed that the changes in platelet complexing had already occurred 1 hour after transfusion. This is in line with a recent publication that demonstrated increased platelet counts 1 hour after elective platelet transfusion in cirrhosis patients.<sup>44</sup> However, this may not allow sufficient time for possible systemic effects of the complexes to occur.

Although several guidelines still recommend platelet transfusion prior to invasive procedures if platelet counts are below  $50 \times 10^9/L$ , suggestions to refrain from this in low risk procedures have been raised.<sup>45–47</sup> The theoretical background for this is the concept of a “rebalanced haemostasis” in cirrhosis, which standard haemostatic tests do not take into account.<sup>48</sup> In this study, we observe improvement in the haemostatic status as measured by Rotem after platelet transfusion, which could favour the transfusion approach. However, the value of Rotem as a global haemostasis test is questionable as it does not take into account changes in VWF and protein C pathway proteins. A recent study demonstrated fewer transfusions with no increase in haemorrhagic incidences when the decision basis for elective platelet transfusion in cirrhosis was guided by thromboelastography.<sup>49</sup> Also, the increases in thrombin-antithrombin complexes with decreases in factor VIII suggest that platelet transfusions result in systemic activation of coagulation with potentially harmful effects, which may include macrovascular thrombosis and microthrombosis within organs. Furthermore, liver disease *per se* increases the risk for transfusion-related acute lung injury (TRALI), which may be explained by platelet transfusion-mediated immune modulation.<sup>50</sup> This study raises the potential issue of the immune priming effect of platelet transfusion, which varies widely in patients with cirrhosis and might lend support to a restrictive approach to platelet transfusion in this setting.

In conclusion, platelet-leucocyte aggregation and the resulting leucocyte activation are enhanced in patients with cirrhosis. Elective platelet transfusion may augment these interactions in susceptible patients and warrants further investigation.

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## SUPPORTING INFORMATION

Additional Supporting Information will be found online in the supporting information tab for this article.

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